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Review

(Re)inventing the Circadian Feedback Loop

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For 20 years, researchers have thought that circadian clocks are defined by feedback loops of transcription and translation. The rediscovery of posttranslational circadian oscillators in diverse organisms forces us to rethink this paradigm. Meanwhile, the original "basic" feedback loops of canonical circadian clocks have swelled to include dozens of additional proteins acting in interlocked loops. We review several self-sustained clock mechanisms and propose that minimum requirements for diurnal timekeeping might be simpler than those of actual free-running circadian oscillators. Thus, complex mechanisms of circadian timekeeping might have evolved from random connections between unrelated feedback loops with independent but limited time-telling capability.

Introduction

Diurnal rhythms in plants and animals were first noticed because of behavioral outputs like leaf movement, activity, or feeding, all of which are synchronized to the geophysical day (Androsthenes, 400 BC). We now know, however, that most of these oscillations persist even in a constant environment (i.e., are "free running") and extend to virtually every aspect of physiology. In mammals, so-called "circadian" clocks regulate sleep-wake behavior, cognition, feeding, heartbeat and blood pressure, renal function, all aspects of digestion and detoxication, and even adult cell division (Gachon et al., 2004). In plants, an equally broad range of both cellular and systems physiology is clock regulated, ranging from cold- and light-dependent responses to nutrient transport and growth patterns (Adams and Carré, 2011); and in photosynthetic bacteria, 30%–60% of the entire transcriptome is under circadian control (Ito et al., 2009; Vijayan et al., 2009), compared to 10% in mammals (Panda et al., 2002; Storch et al., 2002).

Although unicellular bacteria and eukaryotes have cell-autonomous circadian clocks, in mammals, disrupted daily behavior was shown very early to be related to a specific brain region, the suprachiasmatic nuclei (SCN) (Stephan and Zucker, 1972). This region, later proven to be a "master clock" necessary for orchestration of circadian physiology and behavior (Ralph et al., 1990), nevertheless has an independent clock within each of its cells (Welsh et al., 1995). The mechanism of this clock is shared and conserved in nearly all metazoans both in master clock and peripheral tissues (Plautz et al., 1997; Yagita et al., 2001), and, as discussed below, its mechanism strongly resembles that in unicellular organisms. Whether in bacteria, eukaryotic cells, or multicellular organisms, these clocks have been defined by their ability to oscillate free running with a period of approximately 24 hr in the absence of external timing cues, their ability to maintain this period independent of external temperature ("temperature compensation"), and their ability to entrain clock phase to the environment.

In this review, we consider known clocks in both unicellular model organisms and larger multicellular ones and illustrate some common design principles among them. In so doing, we have completely ignored the question of how clocks are entrained by their environment, as well as how different clocks in multicellular organisms communicate with one another (reviewed in Dibner et al., 2010; Golombek and Rosenstein, 2010; Kozma-Bognár and Káldi, 2008; Tomioka and Matsumoto, 2010). Instead, we have chosen to focus on cell-autonomous timekeeping: its various mechanisms, how it might have evolved, and what benefits it might confer.

Clock Mechanisms I: Transcription-Translation Feedback Loops

First insight into the molecular mechanisms of how organisms might anticipate daily changes in their environment was proposed for the circadian clock of the fruit fly *Drosophila melanogaster*. Based on the observation that mRNA and protein oscillations of the previously cloned clock gene *Period* are necessary for behavioral rhythmicity in the fruit fly and influence each other, Hardin et al. (1990) proposed a feedback mechanism between mRNA and protein levels. However, whether this was a direct effect of the protein on mRNA transcription or an indirect one through other behavioral or biochemical signals remained unanswered. This question was first resolved in fungi, i.e., the bread mold *Neurospora crassa*, in which the negative feedback of the Frequency (FRQ) protein was shown to autoregulate its own transcription (Aronson et al., 1994). Today, this principle of a transcription-translation feedback loop (TTFL) is considered to be a universal building block of circadian clocks and has been identified in all model systems studied to date.

In mammals, for example, the "core" oscillator was found to be based on the negative feedback of Period (PER1-3) and Cryptochrome (CRY1,2) proteins on their own transcription. This mechanism has been reviewed extensively elsewhere (Ripperger and Brown, 2010). In brief, transcription of *Per* and *Cry* genes is driven by a heterodimer of the activators CLOCK and BMAL1/NPAS2, and PER and CRY proteins interfere with this positive drive. Both activation and repression are accompanied by extensive changes in posttranslational modification of surrounding histones (Brown, 2011), and the stability and the activity of these clock proteins are also controlled by posttranslational modification (Kojima et al., 2011), discussed further below.

In *Drosophila*, a very similar mechanism is used: PER and TIM proteins (this time each represented by a single gene) repress their own transcription. This transcription is activated by CLOCK

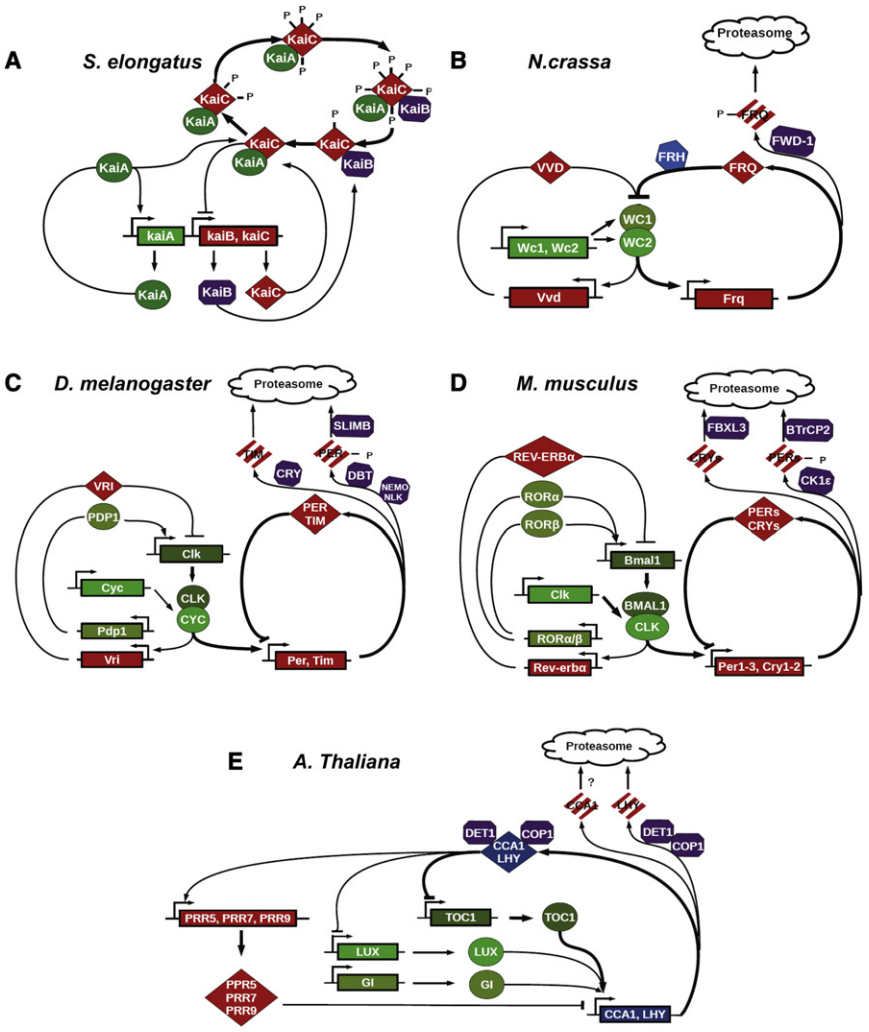

Figure 1. Mechanisms of Circadian Clocks in Different Organisms

Schematic outline of canonical circadian feedback loops in *Synechococcus elongates* (A), *Neurospora crassa* (B), *Drosophila melanogaster* (C), *Mus musculus* (D), and *Arabidopsis thaliana* (E). In each case, a principal loop (bold lines) is supported by interlocked parallel loops (lighter lines) sharing common components, either activators (green) or repressors (red). In cyanobacteria (A), the principal loop is a posttranslational feedback loop (PTFL), based on cycles of KaiC phosphorylation. In the four eukaryotes, the primary known loop is a transcription-translation feedback loop. Recent research suggests that all five systems (A–E) might also contain PTFL-based machinery regulating protein oxidation.

and CYCLE (orthologs of CLOCK and BMAL1 in mammals). The CRY protein here functions as a blue-light photoreceptor that interacts with TIM to promote its degradation, as well as modulating transcriptional activity like mammalian CRYs (Hardin, 2011). In other insect species, a CRY2-like protein with a primary function as transcriptional repressor also exists. The TIM protein, though essential to clock function in flies, is not conserved in mammals. (The mammalian TIM protein is a closer homolog of the *Drosophila* gene *Timeout*, and its function in the circadian oscillator is controversial [Tomioka and Matsumoto, 2010].)

In *Neurospora crassa*, transcription of the *Frq* gene is driven by the WHITE COLLAR (WC) complex, comprised of the proteins WC1 and WC2. Subsequently, the FRQ protein interacts with an RNA helicase, FRH, and this complex represses *Frq* transcription. The blue-light photoreceptor VIVID acts similarly to *Drosophila Cry* to promote clock protein degradation and modulate transcription, but this time interacts with both the repressing FRQ/FRH complex and the positive WC complex. Although the proteins of the *Neurospora* clock are not directly conserved among *Drosophila* and mammals, they share homologies within certain domains, such as the PAS (Per-Arnt-Sim) domain found in PER, CLOCK, WC1-2, and VIVID. More importantly, the core TTFL feedback loop structure is very similar (Baker et al., 2011). A similar feedback loop structure is also found in plants, where the positive factor TOC1 is repressed by negative factors LHY and CCA1 (McWatters and Devlin, 2011). In fact, the same feedback is even conserved in the cyanobacterium *Synecchococcus aureus*, in which the KaiA protein activates transcription of the *KaiBC* operon, and the KaiC gene product represses it (Ishiura et al., 1998). These loops are schematically summarized in Figure 1.

Clock Mechanisms II: Posttranslational Feedback Loops

For both prokaryotic and eukaryotic clocks, more and more evidence suggests that clock speed is determined by posttranslational modifications such as phosphorylations in plants as well as fruit flies and mammals (Chiu et al., 2011; Isojima et al., 2009; Lee et al., 2011; Más et al., 2003; Terauchi et al., 2007). Therefore, not only is the abundance of transcripts and proteins clock controlled, but phosphorylation states of some components also change rhythmically. Multiple other circadian posttranslational modifications, either of clock proteins or of histones surrounding the *cis*-acting elements to which they bind, have also been identified. These include acetylation (Etchegaray et al., 2003), methylation (Brown et al., 2005), sumoylation (Cardone et al., 2005), and ubiquitination (Naidoo et al., 1999). The kinases and other enzymes that perform these reactions have been identified as important clock components, and the modifications themselves serve a variety of roles, including degradation signals, binding regulators, and signals for recruitment of a variety of additional factors (Kojima et al., 2011).

Surprisingly, in the case of cyanobacteria, regular 24 hr changes in phosphorylation can also be observed in a test tube which contains only the three Kai proteins and ATP to fuel the hydrolytic activity of KaiC that drives this oscillation

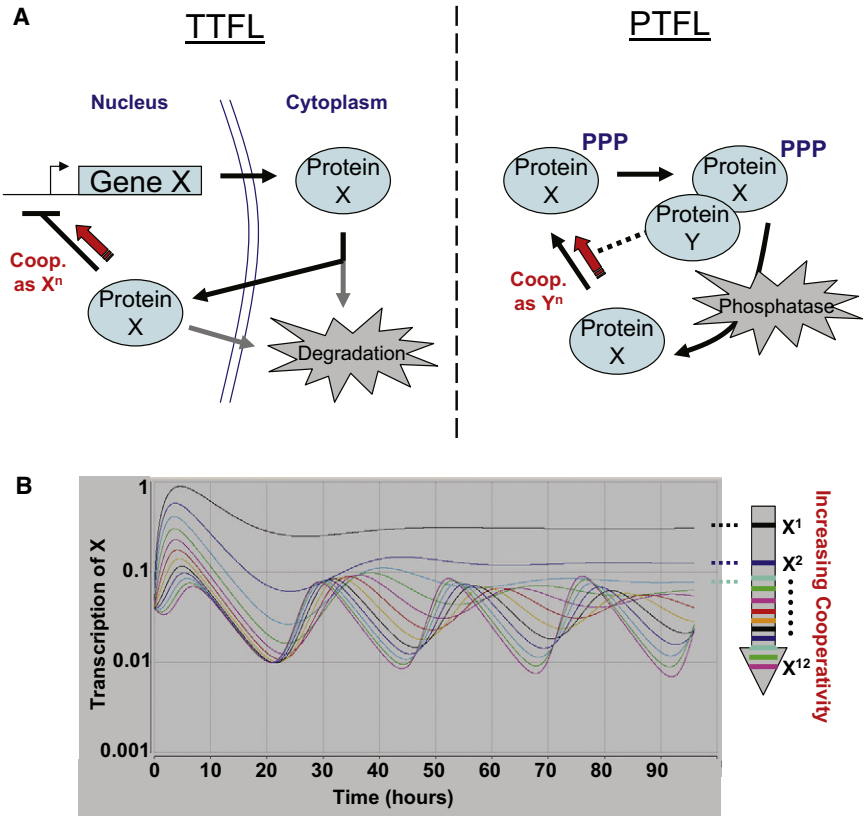

Figure 2. Simplified Clock Models Require Cooperativity

(A) Left: schematic drawing for a transcription-translation feedback loop Goodwin-type oscillator, in which transcription of gene X results in production of a cytoplasmic protein X, which is subsequently imported to the nucleus to repress expression of its gene in a highly cooperative fashion. Right: the same model applied to a post-translational feedback loop, in which phosphorylation of protein X occurs highly cooperatively according to the amount of protein Y, and this phosphorylation is subsequently removed by a phosphatase. Adapted from Axmann et al. (2007).

(B) Cooperativity is essential for sustained oscillation in this simple model. Whereas linear repression (X^1, black line) results in rapid dampening, higher powers (colored lines) result in increasingly robust oscillation. The TTFL model is shown here, with equations and initial coefficients from Ruoff et al. (2001).

(Nakajima et al., 2005). Thus, at least in some organisms, post-translational feedback loops (PTFLs) might be sufficient to explain aspects of circadian function. In the bacterial example cited here, KaiA activates and KaiB represses autophosphorylation of KaiC, a modification that is later hydrolyzed by an internal phosphatase activity also contained within KaiC (Dong et al., 2010) (Figure 1D).

Recently, another circadian posttranscriptional modification—superoxidation of peroxiredoxin proteins (Reddy et al., 2006)—was shown to occur independently of transcription and translation in eukaryotic systems as well. This transcription-independent oscillation was fully documented in mammalian red blood cells (O'Neill and Reddy, 2011) and in the algae *Ostreococcus tauri*, but at least the proteins themselves are conserved in a great number of organisms, suggesting that the clock driving these oscillations might also be widely conserved (O'Neill et al., 2011). The family of peroxiredoxin proteins are antioxidants that prevent damage from reactive oxygen species (Rhee and Woo, 2011). Hyperoxidation of specific family members that is dependent on the redox state of the cell was shown to be rhythmic even in the complete absence of transcription and in the absence of some clock genes necessary to TTFL function. However, to date, it is not clear whether this system influences or regulates known TTFL-based clocks. Moreover, it is also unclear whether the oscillation in oxidative state of the peroxiredoxins is itself part of this new PTFL-based clock, or rather an output of another oscillator yet to be found.

Common Structural Elements between TTFLs and PTFLs

Given the diversity of proteins and processes outlined above in circadian oscillators from different organisms, it would at first glance appear difficult to extract common mechanisms. However, all of these clocks can share the common mathematical framework of a simple negative feedback loop. At least on paper, this structure is intrinsically sufficient to determine biological oscillations, and completely synthetic biological oscillators in bacteria or in eukaryotic cells have been created to demonstrate this point (Elowitz and Leibler, 2000; Tigges et al., 2009). For the circadian oscillator, many different theoretical models have been proposed, ranging from detailed and accurate models which contain differential equations representing synthesis and degradation of each known component with experimentally determined rate constants (Forger and Peskin, 2005; Leloup and Goldbeter, 2011) to more conceptual ones in which classes of proteins and processes are represented by single mathematical terms (Locke et al., 2008; Rougemont and Naef, 2007).

One of the simplest models, and also one of the first to be used, is the Goodwin oscillator. This model was developed in the late 1960s to model free-running biological oscillations of all sorts (not just circadian ones) and postulates that only a clock component, or "state variable," is modified in some fashion, and then this modified form represses the expression or activity of the original component (Goodwin, 1965). In the context of the circadian clock, this description has often been interpreted as a clock protein that is modified or imported to the cell nucleus, where it represses its own expression (Figure 2A). The same model, however, can be used to describe (in simplified form) other clock systems, including posttranslational ones, such as the phosphorylation-based oscillations of the KaiABC system in cyanobacteria (Axmann et al., 2007).

Since its first description, this theoretical framework has been adapted by many different laboratories to reflect free-running

oscillations with a period of about 24 hr (e.g., Ruoff et al., 1999). Systematically varying these parameters is a useful exercise to show the characteristics of a feedback loop that allow it to tell biological time. By doing this, two lessons emerge:

(1) Somewhat counterintuitively, rate constants for degradation are critical to the oscillations of the system, but rate constants of synthesis are far less so (Ruoff et al., 2001). In other words, whereas circadian synthesis rates of clock components can be varied without significant changes in clock properties, degradation rates can only vary within a relatively narrow window while still avoiding perturbations.
(2) Linear repression in simple models does not suffice. Rather, repression must be highly cooperative or "nonlinear," something achieved mathematically by making repression proportional not simply to the concentration of the repressor, but to a power of this concentration. This exponent hovers between 5 and 9. As can be seen in Figure 2B, increasing cooperativity in repression results in increasingly self-sustained oscillations. Beyond this point, although too much cooperativity does not impede oscillation, it does increase sensitivity to perturbation (Saithong et al., 2010).

Although these predictions are derived from a completely abstract and simplified oscillator, their validity can be examined by looking at actual data from more complicated clocks in model organisms, as the next paragraphs illustrate.

Synthesis and Degradation

For example, if synthesis rates are less important, then it ought to be possible to replace circadian transcription of some clock genes by constitutive expression without destroying clock function. This experiment has been performed in *Drosophila* with the predicted outcome that noncircadian expression of the clock proteins PERIOD and TIMELESS still allows circadian oscillations (Yang and Sehgal, 2001). In mammals, similar results have been seen for CLOCK, CRY, and PER proteins (Fan et al., 2007; Yamamoto et al., 2005). More generally, the mammalian circadian clock is resistant to large overall variations in transcription rates at a cellular level (Dibner et al., 2009). Nevertheless, such a simplification has its limits: overexpression of repressive clock factors has proven effective to interrupt oscillator function in both plants and animals (Kornmann et al., 2007; Matsushika et al., 2002).

Additionally, if rate constants for degradation are critical, then one can predict that many cellular components involved in such degradation would be identified as essential components of the circadian oscillator. For metazoan transcription-translation feedback loops, this prediction appears true. Starting with the discovery of PER phosphorylation, ubiquitination, and degradation by the proteasome in mammals and *Drosophila* (Keesler et al., 2000; Price et al., 1998) and of an identical fate for FRQ in *Neurospora* (He et al., 2003), many different components of degradation pathways have been identified as clock components whose activity is necessary for correct circadian oscillations. Indeed, ubiquitin/proteasome pathway components arise as one of the principal classes of new clock proteins identified by recent genome-wide RNA-interference-based screens (Maier et al., 2009; Sathyanarayanan et al., 2008) and ENU mutagenesis screens in mammals (Siepka et al., 2007), and proteasome activity is also essential to the plant-like CCA1-TOC1 feedback loop of the unicellular algae *Ostreococcus tauri* (van Ooijen et al., 2011).

Specifically, it has been shown that the PER proteins in mammals are phosphorylated in a two-step process by Casein Kinase 1ε/δ and possibly other kinases (Vanselow et al., 2006; Xu et al., 2007). One main effect of this phosphorylation is to allow the recognition of PERs by the F-box-containing protein βTrCP2 and its subsequent degradation by the proteasome (Reischl et al., 2007). CRY proteins are similarly regulated by a different F box protein, FBXL3 (Godinho et al., 2007; Siepka et al., 2007). In *Drosophila*, a precisely analogous targeted degradation occurs. The kinase NEMO/NLK acts as a primer kinase to phosphorylate PER, which is later phosphorylated by DOUBLETIME (the *Drosophila* homolog of Casein Kinase 1ε) to permit recruitment of the F box protein SLIMB (a homolog of the mammalian protein bTrCP) and allow degradation of PER by the proteasome (Chiu et al., 2011). In *Neurospora* and in plants, similar proteasome-targeted degradation events have also been shown as crucial to clock function (He et al., 2003) (Figures 1A–1E).

For the cyanobacterial posttranslational clock system, the principal oscillating component identified so far is the phosphorylation of KaiC. In this case, the removal of this phosphoryl group by the intrinsic phosphatase activity of the KaiC protein is essential to setting the pace of the circadian oscillator (Terauchi et al., 2007). However, other targeted degradation systems (phosphatases or proteases) have not been investigated. Similarly, although virtually nothing is known about the reactions that might comprise the mammalian posttranslational circadian oscillator, one can assume that the degradation rates of its components will also be key. If we presume that the posttranslational hyperoxidation of peroxiredoxins is the critical "state variable" of the clock, then we can equally predict that the sulfiredoxin enzyme that catalyzes its reduction will be a critical clock component. (Moreover, whereas mammalian cells have six peroxiredoxins, there is only one sulfiredoxin, so this protein should provide an interesting target for intervention.) Proteasomal degradation, by contrast, is not important for PTFL-based oscillations in transcriptionally silenced *O. tauri* cells, though other forms of regulated degradation have not been eliminated (van Ooijen et al., 2011). Because the peroxidase activity of peroxiredoxins themselves is highly regulated by tyrosine and threonine kinases (Woo et al., 2010), these too might prove to be important clock components. In such a hypothetical loop, the positive component might even be respiratory or mitochondrial function in general, because the electron transport chain of aerobic respiration produces the cellular hydrogen peroxide that normally oxidizes peroxiredoxins (Murphy, 2009).

Cooperative Repression

The second implication evident from mathematical clock models is a need for cooperativity in repression. However, what is the biological meaning of repression proportional to the ninth power of the concentration of the repressor? The short answer is that with a single-component repressive system, it is difficult to

imagine. However, by broadening the system, either linearly to create a larger loop or laterally to interlock feedback loops, the need for cooperativity at any one component is reduced. An analogous problem is resolved by conventional (noncircadian) signal transduction, in which multicomponent cascades are used to create highly cooperative responses to tiny changes in ligand-receptor interactions.

In known TTFLs, the interaction of PER with TIM or CRY in mammals and flies (Gekakis et al., 1995; Griffin et al., 1999) or FRQ with FRH in *Neurospora* (Cheng et al., 2005) is itself a simple form of cooperative repression. More generally, in all studied oscillators, interlocked parallel feedback loops exist. For example, in cyanobacteria, while oscillations can be achieved by strictly posttranslational mechanisms in vitro, they are reinforced by transcription-translation feedback loops running in parallel from the *KaiA/BC* operons, as described above (Kitayama et al., 2008; Qin et al., 2010; Zwicker et al., 2010). In *Neurospora*, the *vivid* locus is transcribed in a fashion dependent upon the WHITE COLLAR complex, and its protein product VIVID itself interacts with FRQ and WCC proteins to modify their activity (Elvin et al., 2005; Hunt et al., 2010). Posttranscriptionally, the exosome is itself regulated in circadian fashion to control *frq* RNA stability in an independent loop (Guo et al., 2009). In plants, the principal TOC1-LHY-CCA1 loop is reinforced by a second loop in which expression of LHY and CCA1 is repressed by PRR7 and PRR9 (Eriksson et al., 2003; Farré et al., 2005) and a third loop in which TOC1 acts to repress expression of GIGANTEA, which itself activates TOC1 (Locke et al., 2006). In *Drosophila*, rhythmic transcription of *Clock* is achieved via a second feedback loop in which this locus is regulated by the VRILLE and PDP1 proteins, whose genes are themselves transcribed in CLOCK-dependent fashion (Cyran et al., 2003).

In mammals, a number of diverse parallel loops have been reported. Within the realm of transcription and translation, the repressor Rev-Erbα, itself activated by CLOCK:BMAL1 heterodimers, is important to repress the transcription of *Bmal1* (Preitner et al., 2002), whereas the homologous ROR nuclear receptors activate it (Sato et al., 2004). Moreover, multiple auxiliary nontranscriptional loops have been reported as essential for correct oscillator function. For example, cAMP-dependent signaling activity is both modulated in circadian fashion and necessary for circadian function (O'Neill et al., 2008). Similarly, NAD/NADH are both regulated by the circadian clock and regulate its activity (Nakahata et al., 2009). In *Drosophila* larvae, ionic currents have also been shown to be important for circadian oscillations in pacemaker neurons (Nitabach et al., 2005). Loops might also be highly indirect: for example, in the cortex, circadian sleep is an important factor for rhythmic expression of a large part of the circadian transcriptome (Maret et al., 2007), and in the liver, circadian feeding plays an equally important role (Vollmers et al., 2009). Such loops could also provide important nodes for environmental influences upon circadian function.

Another way in which the mammalian circadian oscillator might achieve high cooperativity is through reliance upon multiple auxiliary factors for repression. In particular, several recent papers have established a strong role for chromatin modification or, alternatively, posttranslational modification of clock factors themselves by chromatin modifying proteins. For example, in mammals, the negatively acting PER proteins recruit accessory proteins NONO (Brown et al., 2005) and SFPQ (Duong et al., 2011), and at least the latter recruits a histone deacetylase. Via the WDR5 adaptor, PER also recruits circadian histone methylation activity (Brown et al., 2005). The positive factor CLOCK is itself an acetylase of histones and of its partner BMAL1 (Doi et al., 2006; Hirayama et al., 2007) and recruits the histone demethylase JARID1a (whose demethylase activity, interestingly, may not be important for circadian function) (DiTacchio et al., 2011). BMAL1 recruits the histone methylase MLL (Katada and Sassone-Corsi, 2010). Indeed, the entire chromatin environment of clock genes fluctuates between open and repressive in daily fashion (Ripperger and Schibler, 2006). Even in cyanobacteria, which do not possess histones, DNA fluctuates between a compact and open structure daily (Vijayan et al., 2009; Woelfle et al., 2007).

Inducing Delay

One additional issue important to circadian biology is delay. Interestingly, for mathematical models of circadian oscillations, such a delay poses no particular problem. Variation of rate constants permits even the simplest of clock models to achieve a wide range of period lengths. In nature, however, many rate constants are constrained by biological reality. For example, circadian clocks evolved to match a 24 hr day, but the actual steps of a transcription-translation feedback loop or a posttranslational feedback loop are relatively rapid. A synthetic TTFL constructed in bacteria that was based on the same principles of a Goodwin-type oscillator had a period of about 2 hr (Elowitz and Leibler, 2000). To extend the period to 24 hr, one can take various steps. First, from multiple systems, it is clear that one crucial step in establishing period is the rate of degradation of clock components. In the case of the posttranslational loop of cyanobacteria, the very slow autophosphatase activity of KaiC is probably sufficient to guarantee delay (Terauchi et al., 2007). In the case of metazoans, the complex phosphorylation of PER proteins certainly plays a role. For example, initial phosphorylation of *Drosophila* PER by NEMO/NLK actually prevents the later phosphorylation by DOUBLETIME that targets it for degradation (Chiu et al., 2011). Second, it has also been postulated that delayed nuclear localization of PER and CRY is also important: in mammals the peak of PER1 protein in the nucleus is actually 8 hr after the peak of synthesis of its RNA (Yagita et al., 2002). Third, the same use of parallel loops and auxiliary factors that is necessary to achieve cooperativity can also be used to achieve delay. For example, the closely homologous factors NONO and SFPQ have both been found to associate with PER proteins, but whereas one enhanced transcriptional repression in cells, the other seemed to antagonize it (Brown, 2011; Duong et al., 2011).

Evolution of Clocks

Altogether, the circadian oscillator has demonstrated an astonishing complexity and diversity of mechanisms—even, as in this review, when considered from an entirely cell-autonomous perspective. Hence, how did such a structure evolve? It would appear from current evidence that multiple clock mechanisms can exist concurrently. For example, the redox oscillation of peroxiredoxins occurs alongside conventional TTFLs of different

mechanisms in mammalian cells and algae and does not require their function (O'Neill and Reddy, 2011; O'Neill et al., 2011). At the same time, during forward genetic screens in many circadian model organisms designed to uncover mechanisms of known TTFL- and PTFL-based circadian clocks, no hints of this other oscillator were found. The simplest explanation would be that the two can exist independently. Similarly, in cyanobacteria grown under certain conditions, the TTFL oscillation of the *Kai* operon can exist independently of cyclic phosphorylation of KaiC (Kitayama et al., 2008), again suggesting that TTFLs can exist independently of PTFLs. Even looking at only TTFLs, little sequence-based evidence exists for conservation of clock proteins themselves among bacteria, plants, and mammals. Thus, phylogenetics suggests that the circadian system probably evolved multiple times, both because clocks in different organisms use different gene setups, and because apparently different clocks exist within the same organism.

Above, we have suggested that the keys to circadian feedback oscillation lie in careful control of degradation rates and in a high degree of cooperative repression achieved by a highly parallel feedback loop structure. Perhaps information about the origin of clocks might be gleaned if one examines what happens if these parameters are disregarded: i.e., what kind of oscillator is created by a simple feedback loop in which there exists little cooperativity, and components are relatively unstable, but not carefully controlled? As seen in the first curve of Figure 2B, the answer is a highly damped interval timer. Increasing cooperativity results in increasingly sustained oscillations, and degradation rate is a key factor in determining period. Nevertheless, such a damped timer could already be highly useful in a rhythmic environment. Compared to a self-sustained circadian oscillator, it would be highly plastic, would easily permit large phase changes, and, within limits, would be adaptable to multiple periods, although in this case phase angle would not be constant. Thus, it would adapt more easily to seasonal and geophysical changes in day length. Depending upon architecture, a simple low-cooperativity feedback loop would also display some degree of temperature compensation and a wide tolerance of regular diurnal fluctuations in temperature, as well as entrainment by them (Ruoff and Rensing, 1996). Therefore, we suggest that for evolution in a circadian geophysical environment, even a simple feedback loop of limited cooperativity would provide some of the advantages of a full free-running oscillator.

Free-Running versus Damped Oscillators

Of course, such an idea immediately initiates another question: what is the benefit of a complicated free-running circadian oscillator, as opposed to a simpler damped one? In nearly all model organisms in which clocks have been studied, there exists a free-running oscillator. Some mammalian species living in polar regions, such as reindeers and birds, specifically "turn off" their circadian clocks entirely during arctic summer or winter (Lu et al., 2010; Reierth and Stokkan, 1998; van Oort et al., 2005), which would not be necessary if their clocks were damping. Conversely, ample evidence exists both from the real world and the laboratory that lifestyle contrary to one's clock (e.g., shiftwork) carries a price, both in terms of disease risk and of lifespan (Castanon-Cervantes et al., 2010; Klerman, 2005). One benefit of a damping oscillator is that it would phase shift much more quickly, largely eliminating such difficulties. More abstractly, from studies in bacteria (Ouyang et al., 1998), flies (Klarsfeld and Rouyer, 1998; Pittendrigh and Minis, 1972), plants (Dodd et al., 2005; Highkin and Hanson, 1954), and mammals (Wyse et al., 2010), it is clear that having a circadian period adapted to the environmental photoperiod carries benefits in terms of lifespan and evolutionary fitness—the "circadian resonance" hypothesis (Pittendrigh and Bruce, 1959). With a simple damped oscillator, such changes would be easily accommodated. Even cavefish that have lived in constant darkness for millions of years surprisingly retain a circadian clock that displays an approximately 2 day period, suggesting that a free-running clock is intrinsically useful. This clock is no longer synchronized by light, but rather uses food as a timing cue (Cavallari et al., 2011).

Hence, something must be useful in a free-running oscillator. But what? Clocks have been postulated to provide a wide variety of evolutionary benefits: segregation of photosynthesis and nitrogen fixation reactions (Berman-Frank et al., 2001); behavioral roles, either in avoidance of predation (Daan, 1981) or enhancement of memory (Ruby et al., 2008); metabolic functions (Roenneberg and Merrow, 2002); energy storage (Hut and Beersma, 2011); avoidance of cancer and mutations via circadian regulation of the DNA damage response (Sancar et al., 2010; Simons, 2009), cell division (Matsuo et al., 2003; Nagoshi et al., 2004), or cellular redox balance (Asher and Schibler, 2011; Piruzyan et al., 1973); and heterogeneity of stem cell populations (Janich et al., 2011). However, all of these ideas do not require a free-running oscillator in a rhythmic geophysical environment.

Returning to the mathematical model of Figure 2, one can attempt to answer this question by asking what oscillator properties change as cooperativity increases, for example, by incorporating additional feedback loops to reinforce one another. One change—and also a possible explanation for the evolution of free-running oscillators—is increased resilience to external noise. As increasing feedback loops are added to an oscillator, not only does the oscillator damp more slowly, but it also displays increasing resistance to environmental fluctuation. In this respect, to us, the most compelling reason for the evolution of free-running oscillators has nothing to do with their free-running properties at all. Rather, a free-running oscillator shows the best resistance of phase angle to external perturbations. A simple damped oscillator will easily adapt to any period, but the phase angle of its components will vary considerably with random noise. By contrast, a free-running oscillator shows much slower adaptation, but its phase angle—even in the presence of noise—very accurately predicts a cycle matched by its endogenous period. Regardless of what exactly a clock evolved to anticipate, such precision would be useful.

So, how might clocks evolve? As mathematical models show, all that is needed to convert a very simple feedback loop into a free-running clock is a measure of added cooperativity that can be built by the coupling of additional feedback loops (Roenneberg and Merrow, 2002). Such connections might happen randomly in nature between unrelated pathways. We postulate, therefore, that clocks might have started as multiple, very simple damped oscillators, which then achieved resilience and stability by crossregulation from other unrelated pathways. More specifically, some have suggested that clocks might have evolved from

simpler mechanisms to ensure that metabolism was entrained to geophysical parameters or segregated from DNA damage (Roenneberg and Merrow, 2002; Tu and McKnight, 2006). Regardless of the evolutionary rationale, the result of a clock evolved from connected loops would be a mechanism as complex, diverse, and elegant as the one we know.

New Mechanisms and Unexplained Clocks

If clocks evolved by connections of feedback loops, simpler or different feedback loops might also explain why some organisms without canonical clocks nevertheless display clock properties. For example, the yeast *Saccharomyces cerevisiae* was long thought to have no clock at all, until recent evidence demonstrated damped circadian metabolic oscillations. Although no free-running oscillations under constant conditions were observed, the authors nevertheless presented convincing evidence of an underlying clock based upon phase relationships with the diurnal cycle (Eelderink-Chen et al., 2010). In fact, a clock mechanism lacking the cooperativity necessary to be free running would exhibit exactly such behavior. Moreover, other authors have shown ultradian genome-wide metabolic oscillations in yeast (Klevecz et al., 2004; Tu et al., 2005) independent of the cell cycle (Slavov et al., 2011). It is as yet unknown whether these two oscillations share common components.

Even in well-established circadian model organisms, there exist unexplained clocks. In addition to the systems that we have discussed, there exist other oscillations, either damped or free running, that can exist in the absence of canonical clock genes in the same model organisms or can coexist with them. For example, in certain mutant strains of cyanobacteria, promoters driven by different sigma factors can oscillate simultaneously with different periods (Nair et al., 2002). In *Neurospora*, circadian spore formation can still be observed under certain conditions in strains lacking the *Frq* locus that is central to the mechanism of the known clock (de Paula et al., 2006; Dragovic et al., 2002). The mechanism of this Frq-less oscillator remains unknown, though recent evidence suggests that it shares some components with the FRQ/WCC-based clock (Li et al., 2011). Similarly, mice given methamphetamine in the drinking water show rhythmic behavior in constant conditions even in strains lacking a functional clock due to genetic ablation of clock genes or stereotaxic lesion of the SCN (Honma et al., 1987; Mohawk et al., 2009). Finally, mice fed rhythmically show behavioral anticipation of food for some days afterwards, again even in SCN-lesioned or some (but not other) genetically clockless strains (Clarke and Coleman, 1986; Feillet et al., 2006; Pitts et al., 2003).

In addition, a considerable number of observations exist to support the notion that, even in model organisms with TTFL-based clocks, some aspects of circadian physiology are not explained by them. For example, in mammals, red blood cells show circadian ATPase activity (Cornelius and Rensing, 1976), acetylcholinesterase (Mabood et al., 1978), and hemoglobin oxidation (O'Neill and Reddy, 2011). Membranes of *Gonyaulax* independently show circadian physiology (Adamich et al., 1976). When the cell nucleus is removed from the protist *Acetabularia*, circadian physiology continues (Mergenhagen and Schweiger, 1975a; Woolum, 1991). Similarly, addition of transcriptional or translational inhibitors either to *Acetabularia* (Mergenhagen and Schweiger, 1975b) or to eyes of the sea slug *Bulla gouldiana* (Page, 2000) does not block circadian functions. Prior to the discovery of TTFL-based mechanisms, such observations were numerous enough to push some investigators to propose entirely membrane-based clock models related to those established for ultradian rhythmicity of neuronal firing (Njus et al., 1974)..

With the discovery of evolutionarily ancient peroxiredoxins present in all organisms, it is natural to think that the oscillator that drives their redox oscillations might be "the" original oscillator from which all others evolved, or at least a scaffold upon which other oscillators might have evolved (Zivkovic, 2011). However, we feel that the simple phylogenetic diversity of clocks argues against a purely common origin. Rather, it is possible that a diversity of circadian and noncircadian networks might exist within single organisms. Some of them—like peroxiredoxin superoxidation or clock-controlled gene transcription and translation—are remarkably resilient, and others are transient, damping, or only occur under specific circumstances. Discovering these oscillators and, more importantly, learning about the rhythmic physiology that they control, will doubtless be fascinating tasks in the years to come.

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